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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)* The purpose of the project is to employ probes isolated from the LNCaP series of human prostate cancer cells, to probe human cDNA microarrays, so as to investigate genes differentially expressed among these cell lines; prepare from the LnCaP series, particularly the C4-2B cells representing the most advance cancer stage, RNA corresponding to a purified "membrane fraction", expected to contain the mRNAs for cell surface markers or secretory proteins; and employ these probes to probe human cDNA microarrays, to identify genes whose products might form clinical assays for advanced prostate cancer. Work during the period covered included:
(i) Incorporation of the powerful new DNA microarray technology into the project, and its use to investigate differential gene expression in the LNCaP series of human cell lines, representing different stages of prostate cancer. (ii) Studies finally yielding preparation of highly pure membrane fraction from these cells; but resulting in insufficient yield for preparation of DNA microarray probes.

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INTRODUCTION

This project is directed to the problem of androgen-independent prostate cancer, which is a major cause of cancer-related mortality among men. The purpose of the research is to clone, isolate and characterize protein markers that are specifically expressed by advanced stage, androgen-independent prostate cancer.

RESPONSE TO ISSUES RAISED IN REVIEWER'S COMMENTS ON ANNUAL REPORT (SECOND)

The Annual Report (Second) was deemed acceptable as written. However, it was stated on p.3 of the Reviewer's report that "Next year's annual report should take into consideration the Editorial, Contractual, and Technical Issues above." These issues are addressed in this section, as follows:

Format/Editorial Issues: No format/editorial issues were noted in the review.

Contractual Issues: Two contractual issues were raised in the review:

1. As a result of shifts in the work methods, an updated SOW was required from the PI. A new SOW has been prepared, and a letter describing this updated SOW is attached to this report in the form of a separate letter to the Grants Officer. The updated SOW is also reproduced below:

Statement of Work (Updated)

Novel Technology for Cloning Prostate Cancer Cell Markers

The updated overall aim of this project is to employ the powerful new DNA Microarray Technology to investigate differential gene expression among the LNCaP series of human prostate cancer cells. This work involves the following tasks:

Task 1. Employ human cDNA microarrays, plus probes isolated from the LnCaP series of cells, to investigate genes differentially expressed among these cell lines.

Task 2. Prepare from the LnCaP series of human prostate tumor cell lines, particularly the C4-2B line representing the most advanced stage of prostate cancer, RNA corresponding to the "membrane fraction", expected to contain the mRNAs for cell surface markers or secretory proteins.

Task 3. Employ membrane fraction RNA isolated from the LnCaP cell series under Task 2, as a probe of human cDNA microarrays, to identify genes for cell surface markers or secretory proteins expressed specifically by the C4-2B cells, which represent the most advantaged stage of prostate cancer. The products of those genes would then represent candidates for clinical assays for advanced prostate cancer.

2. "Technical problems encountered, particularly with the proteomics project, should be described in more detail." The projected proteomics project involved both the use of the Ciphergen Biosystems SELDI ProteinChip system; and a

collaboration with Dr. Marc Glucksman of the Neurobiology Center of Mount Sinai School of Medicine, to employ his expertise in two-dimensional gel electrophoresis of proteins. In both cases, the object was to identify membrane fraction proteins expressed by the C4-2B cells, but not the LnCaP cells. The following technical problems were encountered:

2A. Ciphergen Biosystems SELDI ProteinChip. As reported in the August, 1999 Annual Report, we began experiments with Ciphergen Biosystems, to carry out protein differential displays. A representative from Ciphergen set up a demonstration SELDI ProteinChip system at Mount Sinai School of Medicine, and employed samples we provided him to carry out several preliminary investigations designed to employ this system to detect differences in the membrane fraction proteins expressed by the C42B and LnCap cells. However, these experiments did not detect any differences in the patterns of membrane fraction proteins expressed by these two cell lines. This negative result was probably due to two technical factors: 1. the low level of protein recovery from the membrane fractions we had isolated from these cells; 2. a low resolution of the SELDI ProteinChip demonstration system that was temporarily set up at Mount Sinai.

2B. Collaboration with Dr. Marc Glucksman. We began this collaboration with Dr. Glucksman because of his expertise in the two-dimensional gel electrophoresis technique; an area in which the Bancroft laboratory has no experience. In the first stage of our projected collaboration, we provided Dr. Glucksman with samples of membrane fractions isolated from the C42B and LnCaP cells. For several months thereafter, when

the PI Dr. Bancroft periodically queried Dr. Glucksman about his progress with the analysis, Dr. Glucksman replied that he was having "technical problems", which he expected to solve in the near future.

Recently, in response to the review of the 2/16/01 Annual Report, Dr. Bancroft has attempted to contact Dr. Glucksman to obtain further details about the technical problems he encountered in his analysis. However, Dr. Glucksman has since moved to the University of Chicago Medical School, and has not responded to a number of messages left on his voicemail there. As a result, it is not presently possible for the PI to provide in this final report, further details about the technical difficulties Dr. Glucksman encountered in his two-dimensional gel analysis of the samples that we provided to him.

Technical Issues: No technical issues were noted in the review.

BODY

I. Successful Propagation of All Four LNCaP-Related Human Prostate Cancer Cell Lines. As described in the August, 1999 Annual Progress Report, we had originally proposed to employ only two related human prostate cancer cell lines in our studies, corresponding to two stages of prostate cancer: the LNCaP androgen-

dependent cells, and their androgen-independent subline, termed C4-2 cells. However, we were actually able to obtain and work with four cell lines in this lineage, corresponding to four major stages in prostate cancer progression: the androgen- and bone-dependent **LnCaP** cells, their bone-independent **C4** cell derivatives, the C4-derivative bone- and androgen-independent **C4-2** cells, and the C4-2-derivative **C4-2b** cells, which exhibit bone metastasis. Although our work with these four cell lines was initially somewhat delayed by difficulties encountered in propagation of these difficult cell lines, we were able finally to propagate and perform investigations with all four of them.

II. Use of DNA Microarray Technology in the Project. As described in the Annual Progress Report of February, 2001, we introduced the cutting-edge and powerful DNA Microarray Technology into this project, with the aim of identifying genes differentially expressed among the above four human prostate cancer cell lines. We purchased from New England Nuclear their MICROMAX microarray system, which includes microarrays containing 2400 known human genes, plus reagents for probe preparation. Use of cDNA probes labeled with either Cy3 or Cy5 permits simultaneous hybridization and analysis on one microarray of probes from two cell lines. The use of reference probes in each hybridization permits normalization of results between experiments. For each cell line, we isolated total RNA, and performed at least two separate hybridizations of a probe prepared from its RNA to a MICROMAX microarray, under conditions where results from each cell line can be normalized and compared.

The result from one such experiment that was analyzed in detail is shown below. In this experiment, gene expression levels were compared between LNCaP cells (Fig. 1A, DNP cDNA label/ Cy3 detection) and C42 cells (Fig 1B, biotin cDNA label/ Cy5 detection). Fig. 1C shows a calculated superposition of the results with the two probes. Following normalization for the two-fold greater mean overall signal yielded by Cy5 relative to Cy3 in this (and most other) experiments, the scatterplot comparing individual gene expression levels shown in Fig.1D was obtained. It can be seen in Fig. 1D that a number of genes are differentially regulated between the two cell lines. Of the 2400 cDNAs present within the NEN microarray, 41 (1.71%) were found to be expressed at significantly higher levels in the LNCaP cell line, while 54 (2.3%) were found to be significantly up-regulated in the C42 cells.

III. Isolation from and Analysis of Membrane and Post-Membrane Fractions from the LNCaP Cell Series.

IIIA. Analysis of fractions isolated by original fractionation scheme. As described in the original application, we have employed the original simple protocol described by Bancroft (1973) to isolate membrane and post-membrane fractions from the LNCaP cell line and two of its sub-lines (C42 and C4-2B). This fractionation scheme has the advantage of employing a minimum number of steps, and thus an expected higher final yield of RNA from each fraction. We then employed reverse transcription, followed by polymerase chain reaction (RT-PCR), to prepare cDNA from RNA isolated from each

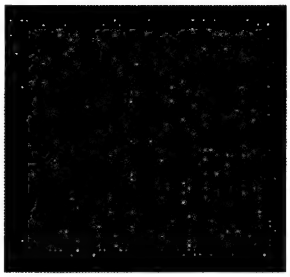
fraction, to examine the intracellular location of the mRNAs for four genes that have the following properties: 1. Either prostate- or tumor-specific expression, and/or potential as a malignancy marker: 2. A protein product either known or believed to be either secreted by or displayed on the surface of prostate cells. These four proteins are:

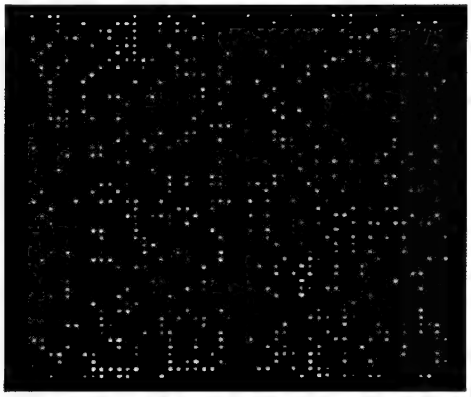
- **PSA (**prostate specific antigen), secreted specifically by the prostate]Urol. Clin. North. Am., Vol. 24 (1997)].
- **PSM (**prostate-specific membrane antigen), displayed specifically on the surface of prostate cells (Israeli et al, 1997).
- **TIMP-2**, a serum metalloproteinase inhibitor that plays a role in tumor invasion, and has been proposed as a potential marker of malignant potential (Kugler et al, 1999). TIMP-2 is expressed by various cell types. The presence of this protein in body fluids (as well as tissues) (Gomez et al, 1997) suggests that it is a secretory protein
- **PAGE-1,** the protein product of a gene recently cloned from the LNCaP cell series, that is testes-specific in normal tissues, and is expressed in a variety of human tumor cell lines. PAGE-1 is believed (but not yet demonstrated) to be expressed as a cell surface antigen (Chen et al, 1998).

The results of our analysis of the intracellular location of the mRNAs for these genes in the LnCaP human tumor cells and their derivatives are shown in **Figure 2.**

Figure 1. Microarray Analysis of Differential Gene Expression between LNCaP and C42 cell lines. 1A) TIF file of original Cy3 scan showing hybridization of DNP-labeled LNCaP cDNA. 1B) TIF file of original Cy5 scan showing hybridization of biotin-labeled C42 cDNA. 1C) ImaGene™ superimposed file of Cy3 and Cy5 scanned images.

1A 

1B 

1C 

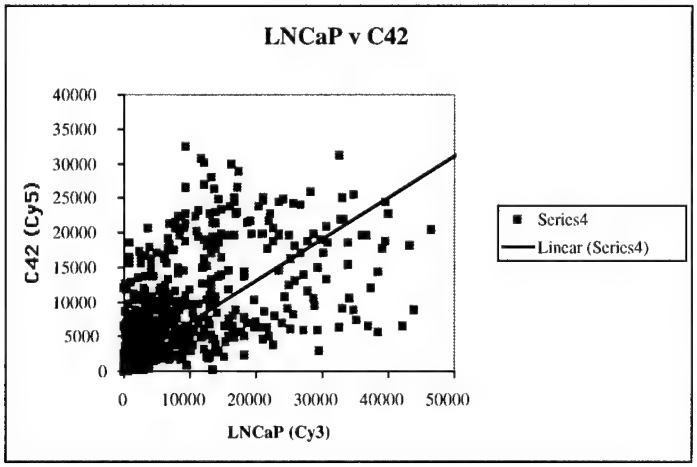
COLOUR CODE KEY:

Original TIF images.
Blue- Green- Yellow- Red-White
→
Transcript Levels

Superimposed TIF images.
Red Cy3 > Cy5
Yellow Cy3= Cy5
Green Cy3< Cy5

Superposition of TIF images in 1A and 1B above, prior to normalization.

1D



Scatterplot of Cy3 (X axis) versus Cy5 (Y axis) signal intensities following normalization.

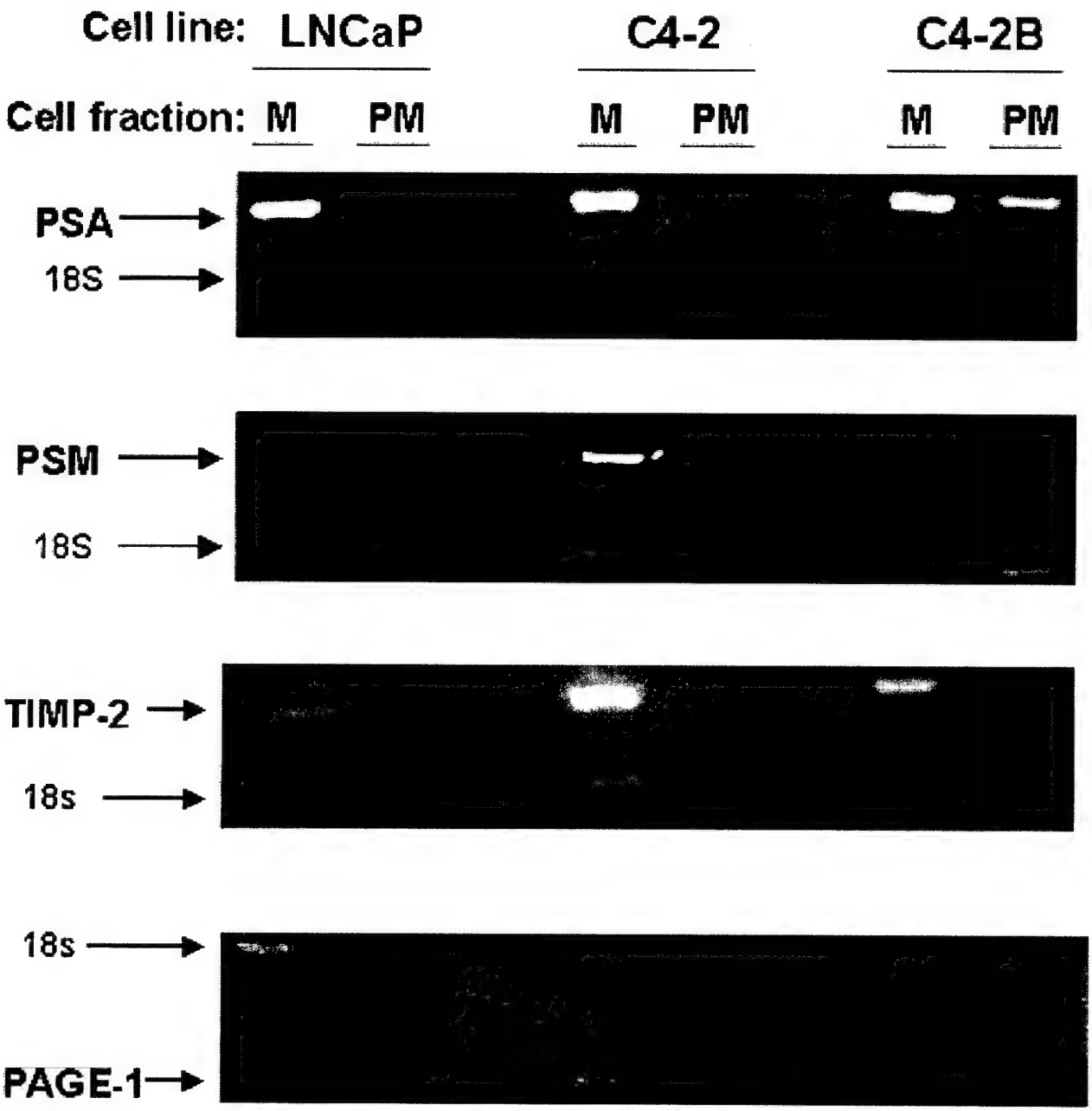


Figure 2. Intracellular location of the mRNAs for genes associated with prostate cancer. Membrane and post-membrane fractions were prepared from the LNCaP human tumor cell line, and its C4-2 and C4-2B derivatives as described (Bancroft [1973] Exp. Cell. Res. 79:275). RNA isolated

from each fraction was reverse-transcribed with random hexamers. The products were then subjected to PCR analysis for each of the indicated genes, employing in each analysis primers specific for each gene, plus primers for 18S ribosomal RNA to serve as an internal control for amount and quality of input RNA. For each gene, a number of cycles determined previously to be in the linear range of amplification was employed. PCR products were analyzed on agarose gels containing EtBr, photographed, and scanned. Amplification products of the expected size were obtained for each gene and for 18S ribosomal RNA. Abbreviations: M and PM, PCR products observed following RT-PCR amplification of RNA isolated from, respectively, the membrane and post-membrane fractions of the indicated cell lines.

The mRNA for the secretory protein PSA was detected in all three cell lines, and was located almost exclusively in the membrane fraction. Only the C4-2 cells exhibited high levels of the mRNA for the cell-surface marker PSM, in which this mRNA is also located almost exclusively in the membrane fraction. This demonstration of the expected intracellular location of the mRNAs for two well-characterized prostate markers, one secreted and one displayed on the cell surface, provides support for the accuracy of the assay we have developed. TIMP-2 mRNA was detected in all three cell lines, again almost exclusively in the membrane fraction. This observation supports the reports discussed above, suggesting that TIMP-2 is a secretory protein, and further validates our assay. Finally, although the gel photograph did not reproduce well, the mRNA for PAGE-1 was found to be expressed significantly only in the C4-2 cells, where its mRNA was preferentially localized in the membrane fraction. This result supports the suggestion, described above, that PAGE-1 is displayed on the surface of prostate cancer cells.

These results were encouraging, since the mRNAs for the four markers were in the expected cellular fraction, the membrane fraction. However, it was necessary also to examine the degree to which the membrane fraction, as we have isolated it, is

contaminated with the post-membrane fraction. The latter fraction should contain mRNAs for proteins that are intracellular, and thus neither secretory nor cell surface proteins, and would thus ultimately yield false positives in our assay. To investigate this potential problem, we have carried out a number of control experiments of the type described above, in which PCR of the cDNAs prepared from mRNA in each fraction was employed to determine the intracellular location of the mRNAs for the following proteins expected to be intracellular: the PSM alternative splice variant PSM' (PSM-prime), which lacks the transmembrane attachment signal sequence (Sai et all, 1995); the transcription factor PREB (Fliss et al, 1999); and the transcription factor CTCF, which maps to a region commonly deleted in prostate (and breast) cancers (Filippova et al, 1998). Unfortunately, we have found that the membrane fractions isolated from the LNCaP cell series by the technique originally proposed and employed yields significant contamination by some or all of these mRNAs.

IIIB. Analysis, and use as a source of probes, of membrane fractions isolated by a scheme employing a sucrose gradient step. Because of this contamination of membrane fractions isolated according to the original scheme, we proceeded to investigate the use of an alternative, more extensive purification technique involving sucrose gradient density ultracentrifugation (Mechler, B.M., 1987) to isolate membrane fractions from members of the LNCaP series. In our initial investigation of the purity of these fractions, we employed RT-PCR analysis of the

mRNAs for the transcription factor CTCF (expected to be in the post-membrane fraction) and PSA and TIMP-3 (expected to be in the membrane fraction). When the RNA was analyzed by the semi-quantitative reverse transcriptase PCR (RT-PCR), this modified fractionation procedure apparently yielded variable success with the cell lines examined, as follows. The best results were obtained with the cell line corresponding to the most advanced prostate cancer stage, the C4-2B cells. With these cells, PSA and TIMP-2 mRNA were again enriched in the membrane fraction; while CTCF mRNA was present in the post-membrane fraction, but was virtually absent from the membrane fraction. The purity of membrane preparations from the C4-2 cells was improved, but these preparations still contained slight contamination with CTCF mRNA. With the LNCaP cells, introduction of this new procedure was found to yield no improvement in the previously detected contamination of the membrane fraction with CTCF mRNA.

Mount Sinai has recently established a facility for performing real-time PCR analysis, a quantitative technique that is far more accurate than the original semi-quantitative PCR assay. We have thus begun to employ an ABI Prism 7700 Real-Time PCR system to reanalyze the mRNAs in the cell fractions described in the preceding paragraph; with an emphasis on analysis of contamination by CTCF of membrane fraction from the C42-B cells, that had been isolated by the sucrose gradient fractionation scheme. Our results have been quite encouraging, with an example shown in **Fig. 3**. It is seen that membranes prepared by the original Bancroft method contained readily detectable levels of CTCF mRNA, while in membranes prepared by the sucrose

gradient centrifugation, CTCF mRNA was virtually undetectable. The latter fraction still contained mRNAs for secretory protein, since real-time PCR analysis yielded readily detectable levels of PSA mRNA (data not shown.)

We had hoped to be able to employ the membrane fraction from the C4-2B cells (as well as the other cells in the LNCaP series) as the source of a probe for DNA microarray experiments of the type described in Section II of the BODY of this report; with the ultimate aim of identifying genes encoding secretory or cell surface proteins, whose expression exhibit changes in gene expression during the tumor progression represented by the LNCaP cell series. However, we have found that, as a result of introducing the sucrose gradient step into the membrane purification procedure, the yield of RNA from this highly purified membrane fraction is too low to permit reproducible preparation of sufficient probe concentrations for use in microarray analysis. It seems probable that this yield problem could ultimately be circumvented by employing techniques developed for preparation of cDNA from very small amounts of starting material (e.g., Kacharmina et al (1999).

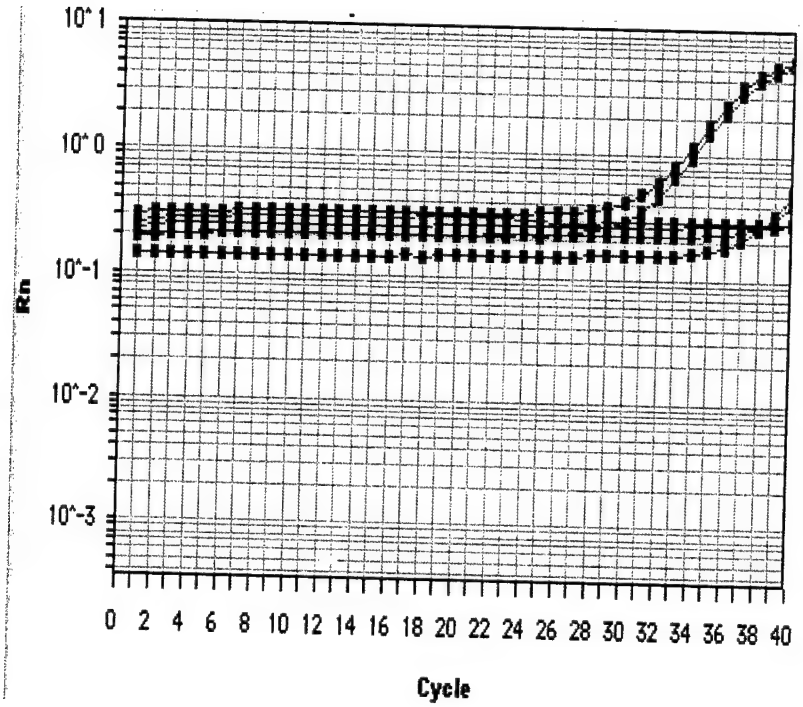


Figure 3. Analysis by Real-time PCR of CTCF mRNA in C4-2B cell membrane fraction, that had been isolated by either of two techniques. The membrane fraction was isolated from C4-2B cells by either sucrose gradient centrifugation (Mechler, B.M., et al [1987] Methods Enzymol. 152:241-248), or by the original Bancroft method (Bancroft [1973] Exp. Cell. Res. 79:275). cDNA was prepared from RNA from this fraction, analyzed in duplicate by real-time PCR with primers specific for CTCFR, and the products detected with SYBR green. Normalized SYBR green signal (Rn) is shown plotted vs. PCR Cycle (Cycle). The samples analyzed were: no template control (shown in blue), or cDNA from RNA isolated from membrane fraction prepared either by sucrose gradient centrifugation (shown in green) or by the original Bancroft method (shown in red).

KEY RESEARCH ACCOMPLISHMENTS

- Incorporated the powerful new DNA Microarray technology into project research, and used it to begin to identify genes differentially expressed among the LNCaP series of human prostate cancer cells. This is apparently the first study with DNA

Microarray technology of differential gene expression among the LNCaP cell series.

- Discovered that membrane fractions previously isolated from these cells exhibited potential utility, but were of insufficient purity for proposed use. Employed an alternative cellular fractionation scheme, involving sucrose gradient centrifugation. Analysis by the accurate real-time PCR method of cDNA made from RNA prepared from sucrose gradient-isolated membrane fractions of these cells showed a virtual absence from these fractions of contaminating mRNA encoding an intracellular protein. Solution of the low RNA yield problem encountered with these highly purified membrane fractions should permit probe preparation from the RNA, and its use as a DNA microarray probe for identification of genes encoding secretory or cell surface proteins, that may be differentially expressed during prostate cancer progression.

REPORTABLE OUTCOMES

We have reported in abstract form the DNA Microarray assay results described above in BODY, Section II (Taylor Clelland et al, 2000).

CONCLUSIONS

Under this grant, we have helped to establish at Mount Sinai the new but technically complex DNA Microarray technology, and have succeeded in employing this powerful technology to pursue the overall goals of this project. Thus, as described above, we have employed this technology to carry out what is apparently the first study of differential gene expression among the LNCaP series of human prostate cancer cells.

We were initially disappointed when further control studies indicated that the mRNAs in the membrane fractions we had previously isolated from these cell lines, although enriched for mRNAs for known prostate cancer markers, were also contaminated with post-membrane fraction mRNAs. However, we have gone on to employ an alternative fractionation scheme, employing sucrose gradient density centrifugation, to isolate a highly pure membrane fraction from the C4-2b cells, which model advanced stage prostate cancer, as well as the other cell lines. As described above in the BODY of this report, we have to date obtained a low yield of RNA from these more highly purified membrane fraction. However, in the future, use of techniques for preparing cDNA from very small amounts of starting material should permit synthesis, from RNA from these highly purified membrane fractions, of DNA microarray hybridization probes specific for mRNAs encoding either secreted or cell-surface proteins. The results obtained with these probes, when compared to the results of the microarray experiments we have already carried out, could ultimately permit

identification of genes that may prove clinically useful in diagnosis/treatment of advanced stage prostate cancer.

PERSONNEL

F. Carter Bancroft,, Catherine Taylor Clelland, Frank Guarnieri, Brian Bloom, Michitaka Kawata

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